Process for producing recombinant human serum albumin.

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Abstract

A process for producing recombinant human serum albumin is disclosed, which comprises culturing a human serum albumin-producing host, prepared by gene manipulation techniques in a medium that contains an amino acid, preferably at least one amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, histidine, serine, tryptophan, valine, isoleucine, phenylalanine, cysteine and arginine, more preferably histidine. The process can significantly increase the yield of human serum albumin over that produced by known processes.

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(54) Process for producing recombinant human serum albumin.

(57) A process for producing recombinant human serum albumin is disclosed, which comprises culturing a human serum albumin-producing host, prepared by gene manipulation techniques in a medium that contains an amino acid, preferably at least one amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, histidine, serine, tryptophan, valine, isoleucine, phenylalanine, cysteine and arginine, more preferably histidine. The process can significantly increase the yield of human serum albumin over that produced by known processes.

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FIELD OF THE INVENTION

This invention relates to the improvement of a process for producing recombinant human serum albumin (to be referred to as "HSA" her inafter) by culturing a host transformed by m ans of gene manipulation techniques.

BACKGROUND OF THE INVENTION

HSA is a main component of plasma proteins and is used in pharmaceutical preparations for the treatment of massive hemorrhage, shock, burn injury, hypoproteinemia, fetal erythroblastosis and the like.

Currently, HSA is produced mainly as a product from fractions of collected blood. However, such a production process is economically disadvantageous, and the supply of blood is sporadic. In addition, blood itself is also problematic in that it often contains undesirable substances such as hepatitis virus.

In order to solve such problems, attempts have been made to produce HSA by fermentation of microorganisms in which the HSA gene was introduced, thereby making use of the recently developing recombinant DNA techniques. However, even such recombinant DNA techniques cannot give satisfactory results in terms of the industrial mass production of HSA.

As a consequence, great concern has been directed toward the establishment of an industrial process for producing recombinant HSA in a large amount with a low cost.

SUMMARY OF THE INVENTION

An object of the present invention is to increase productivity of recombinant HSA in a process for the production thereof by means of gene engineering techniques, particularly to provide a large scale HSA production process which is effected by simple changes in the culture conditions of an HSA-producing host.

As a result of intensive investigation in order to solve the above-described problems, the present inventors have found that productivity of HSA can be increased when an HSA-producing host, prepared by gene manipulation techniques, is cultured in an amino acid-containing medium.

Thus, the present invention relates to a process for producing recombinant HSA which comprises culturing an HSA-producing host, prepared by gene manipulation techniques, in an amino acid-containing medium. More particularly, the present invention relates to a process for producing recombinant HSA, comprising:

(1) preparing a culture medium containing at least one amino acid selected from the group consisting of alanine (to be referred to as "Ala" hereinafter), aspartic acid (to be referred to as "Asp" hereinafter), arginine (to be referred to as "Arg" hereinafter), cysteine (to be referred to as "Cys" hereinafter), glutamic acid (to be referred to as "Glu" hereinafter), histidine (to be referred to as "His" hereinafter), isoleucine (to be referred to as "Ile" hereinafter), phenylalanine (to be referred to as "Phe" hereinafter), serine (to be referred to as "Ser" hereinafter), tryptophan (to be referred to as "Trp" hereinafter) and valine (to be referred to as "Val" hereinafter), and (2) culturing an HSA-producing host in said culture medium.

In another embodiment of the invention, the culture medium contains at least one amino acid selected from the group consisting of Ala, Asp, Glu, His, Ser, Phe, Trp and Val.

In a further embodiment of the invention, the culture medium contains at least one amino acid selected from the group consisting of Ala, Asp, His, Ser, Trp and Val.

In an additional embodiment of the invention, the culture medium contains at least one amino acid selected from the group consisting of Ala, Arg, His, Phe, Ser, Trp and Val.

In another embodiment of the invention, the culture medium contains His.

The culture medium contains the amino acid(s) as described in the media above in an amount of from 0.08 to 20 w/v %.

Additionally, the HSA-producing host may be a yeast strain.

DETAILED DESCRIPTION OF THE INVENTION

The HSA-producing host to be used in the pr sent invention is not particularly limited, provided that it is a cellular host prepared via gene manipulation techniques. Any of the hosts disclosed in published reports, and thos which will be developed in the future may be us d. Illustrative examples of such hosts include microorganisms (*Escherichia coli*, a yeast strain, *Bacillus subtilis* and the like), as well as animal cells, which have been made into HSA-producing cells by gene manipulation techniques. According to the present invention, it is desirable to use as the host a strain of yeast, especially belonging to th genus

Saccharomyces, such as *Saccharomyces cerevisiae*, the genus *Pichia*, such as *Pichia pastoris*, the genus *Kluyveromyces*, such as *Kluyveromyces lactis* or the genus *Hansenula*, such as *Hansenula polymorpha*. An auxotrophic strain or an antibiotic sensitive strain may also be used. Specific examples thereof include G418 sensitive strains such as *Saccharomyces cerevisiae* AH22 (*a*, *his* 4, *leu* 2, *can* 1), *Pichia pastoris* GTS115 (*his* 4) and *Kluyveromyces lactis* MW98-8c (α, uraA, arg, lysK$^+$, pKD1^0). It is preferable to use *Pichia pastoris*, particularly *Pichia pastoris* GTS115.

Preparation of HSA-producing host, production of HSA by its culturing and isolation and recovery of HSA from the cultured broth are all carried out in accordance with known methods which may be modified slightly. For example, preparation of an HSA-producing host (or an HSA-producing strain) may be effected using a process in which a natural human serum albumin gene is used (JP-A-58-56684 corresponding to EP-A-73646, JP-A-58-90515 corresponding to EP-A-79739 and JP-A-58-150517 corresponding to EP-A-91527), a process in which a modified human serum albumin gene is used (JP-A-62-29985 and JP-A-1-98486 corresponding to EP-A-206733), a process in which a synthetic signal sequence is used (JP-A-1-240191 corresponding to EP-A-329127), a process in which a serum albumin signal sequence is used (JP-A-2-167095 corresponding to EP-A-319641), a process in which a recombinant plasmid is introduced into a chromosome (JP-A-3-72889 corresponding to EP-A-399455), a process in which hosts are fused (JP-A-3-53877 corresponding to EP-A-409156), a process in which a mutation is generated in a methanol containing medium, a process in which a mutant AOX_2 promoter is used (EP-A-506040), a process in which HSA is expressed in *B. subtilis* (JP-A-62-215393 corresponding to EP-A-229712), a process in which HSA is expressed in yeast (JP-A-60-41487 corresponding to EP-A-123544, JP-A-63-39576 corresponding to EP-A-248657 and JP-A-63-74493 corresponding to EP-A-251744) and a process in which HSA is expressed in *Pichia* (JP-A-2-104290 corresponding to EP-A-344459). (The term "JP-A" as used herein means an "unexamined published Japanese patent application")

Of these methods, the method in which mutation is induced in a methanol-containing medium is carried out in the following manner.

A transformant of an appropriate host, preferably a *Pichia* yeast, illustratively a strain GTS115 (NRRL deposition No. Y-15851), is obtained in the usual manner by introducing a plasmid, containing a transcription unit by which HSA is expressed under the control of the AOX_1 promoter, into the AOX_1 gene region of the host (cf. JP-A 2-104290). This transformant hardly grows in a medium containing methanol. In consequence, this transformant is cultured in a methanol-containing medium to generate mutation, and a strain capable of growing in the medium is isolated. Methanol concentration in the medium may range, for example, from 0.0001 to 5%. The medium may be either synthetic or natural. The culturing may be carried out, for example, at a temperature of from 15 to 40 °C for approximately from 1 to 1,000 hours.

Culturing of the HSA-producing host (namely the method for the production of HSA) may be effected by each of the methods disclosed in the above patents, by a method in which producer cells and the product are obtained in high concentrations by a fed-batch culture which method is carried out by gradually supplying a high concentration solution of glucose in appropriate small amounts to avoid high concentration substrate inhibition against the producer cells (JP-A-3-83595), by a method in which the HSA productivity is improved by the addition of fatty acids to the culture medium (JP-A-4-293495 corresponding to EP-A-504823 and U.S. Patent 5,334,512) or by a method in which coloring of HSA is inhibited by culturing a host in the presence of diamines and the like (JP-A-5-260986 corresponding to EP-A-591605 and U.S. Patent 5,369,020).

The medium to be used in the production process of the present invention is an amino acid-containing medium, especially a medium which contains at least 1 amino acid selected from neutral amino acids such as glycine (to be referred to as "Gly" hereinafter), Ala, Ser, Val, leucine (to be referred to as "Leu" hereinafter), Ile, Cys, Phe, Trp or Proline (to be referred to as "Pro" hereinafter), acidic amino acids such as Asp or Glu and basic amino acids such as Arg or His. An example of a medium capable of markedly increasing HSA productivity when used in the culturing of an HSA-producing host is a medium which contains at least 1 amino acid selected from the group consisting of Ala, Arg, Asp, Glu, Gly, His, Phe, Ser, Trp, Cys, Ile and Val, preferably a medium which contains at least 1 amino acid selected from the group consisting of Ala, Asp, Glu, His, Phe, Ser, Trp and Val, more preferably a medium which contains at least 1 amino acid selected from the group consisting of Ala, Asp, His, Ser, Trp and Val. On the other hand, in comparison with an amino acid-fr medium, a medium which contains at least 1 amino acid selected from the group consisting of Ala, Arg, His, Ser, Trp, Phe, and Val is effective in producing HSA in a large quantity without allowing an HSA-producing host its lf to grow when used in the culturing of the host. Production of a large quantity of HSA by not allowing the host cells themselves to grow significantly is advantageous especially in the case of an expression system in which HSA is allowed to secrete into culture supernatant, because the ratio of culture supernatant to the amount of culture broth (medium)

becomes large so that HSA can be recovered in a higher yield.

A medium which contains His is particularly pr ferred as the medium to be used in the present invention. This medium is especially good for improving productivity of HSA, because it can considerably increase production yield of HSA independent of the growth of the HSA-producing host its lf.

The medium to be used in the present invention may contain th_ abov_-d_scribed amino acid alon_ or as a mixture of two or more amino acids.

The amino acid content of the medium may range, for example, from about 0.08 to 20 w/v %, preferably from about 0.1 to 1 w/v %.

Other components of the medium to be used in the present invention are not particularly limited, provided that the medium contains at least one of the above-described amino acids. Examples of other components are those which are contained in known culture media generally used in this field. In general, various sugars are used as carbon sources, urea, ammonium salts, nitrates and the like are used as nitrogen sources and various vitamins, nucleotides and the like are used as trace nutrients, as well as inorganic salts such as of Mg, Ca, Fe, Na, K, Mn, Co, Cu and the like.

Illustrative examples of the useful medium include YNB liquid medium (0.7% Yeast Nitrogen Base without amino acids (manufactured by Difco) and 2% glucose), MeOH-ammonium acetate medium (composition: cf. Examples), YPD liquid medium (1% Yeast Extract (Difco), 2% Bacto-peptone (Difco) and 2% glucose) and the like. When the HSA-producing host is a methanol assimilating strain, a methanol-containing medium may be used. In that case, the methanol concentration may range approximately from 0.01 to 5%.

In other words, the medium to be used in the present invention can be prepared easily, by adding the above-described amino acid(s) to any known medium.

The pH of the medium may be neutral, slightly basic or slightly acidic. Preferably, the medium may have a pH value of from about 5.7 to 6.5.

Culture conditions may be selected in the usual way.

The culture temperature may range, for example, generally from about 15 to 43 • C. It may range from about 20 to 37 • C when the host is a bacterium. It may range from about 20 to 30 • C when the host is a yeast. Particularly, the yeast host may be cultured at a temperature of generally from 21 to 29 • C, preferably 21 to 28 • C, more preferably 23 to 28 • C, most preferably 25 to 27 • C. The culture period is approximately from 1 to 1,000 hours.

It is desirable to carry out a seed culturing prior to the main culturing making use of, for example, the above-described YNB or YPD liquid medium. Preferably, the seed culturing may be carried out for, for example, about 10 to 100 hours preferably at about 30 • C in the case of yeast strains or about 37 • C in the case of bacterial strains. The above-described amino acid(s) may also be used in the seed culturing.

After completion of the culturing, HSA is collected from the culture supernatant (filtrate) or cells by isolation and purification means commonly known. Illustrative examples of such means include a method in which HSA is purified by subjecting a culture supernatant (filtrate) to ultrafiltration, heat treatment, acid treatment and ultrafiltration, in that order, and then to respective treatments with cation exchanger, hydrophobic chromatography and anion exchanger (JP-A-5-317079 corresponding to EP-A-570916) and a method in which HSA is decolorized by chelate resin treatment (JP-A-5-328991 corresponding to EP-A-570916).

The following examples are provided to further illustrate the present invention, but are not to be understood as limiting the scope of the present invention.

EXAMPLE 1

(1) Preparation of strain to be used

A strain of *Pichia pastoris*, PC4130, has been obtained in accordance with the method disclosed in JP-A-2-104290, by substituting the AOX_1 gene region of *P. pastoris* GTS115 (*his* 4) with a *Not*I-digested fragment of plasmid pPGP1 which contains a transcription unit by which HSA is expressed under the control of the AOX_1 promoter. Because of the absence of the AOX_1 gene, this strain has a reduced ability to grow on a medium which contains m thanol as the carbon source (methanol assimilation negative strain; to be referred to as "Mut$^-$ strain" hereinafter).

The strain PC4130 was inoculated into 3 ml of YPD medium (1% yeast extract, 2% Bacto Peptone and 2% glucose). After 24 hours of culturing, the cells were inoculated into 50 ml of YPD medium so that the cell density was adjusted to initial turbidity with an OD_{540} of 0.1. After 3 days of culturing at 30 • C, the resulting cells again were inoculated into 50 ml of YPD medium at an initial cell turbidity of 0.1 at OD_{540}.

Thereafter, subculturing was repeated every 3 days in the same manner. After each subculturing, cells were diluted with sterile water and poured onto a 2% MeOH-YNBw/oa.a. plate (0.7% Yeast Nitrogen Base without Amino Acids, 2% methanol and 1.5% agar powder) in an inoculum size of 10^7 cells/plate, followed by 5 days of culturing at 30 • C to judge the presence/absenc of colonies. Twenty colonies were found on the 2% MeOH-YNBw/oa.a. plate after 12 days of the successive subculturing. Mut^- strains hardly grow on the 2% MeOH-YNBw/oa.a. medium, while Mut^+ strains (methanol assimilation positive strains) grow well. That is, the presence of a colony means that the strain acquired the capacity of increased methanol assimilation and thus a Mut^+ strain was obtained. One of the thus obtained colonies was diluted appropriately with sterile water and spread onto a 2% MeOH-YNBw/oa.a. plate to isolate single colonies. One of the resulting single colonies was named GCP101.

An HSA expression plasmid pMM042 was constructed using an AOX_2 promoter (a mutant of the natural AOX_2 promoter (*YEAST*, 5, 167-177, 1988; *Mol. Cell. Biol.* , 9, 1316-1323, 1989), in which the 255th base upstream from the initiation codon of said promoter is changed from T to C) isolated from the above-described strain GCP101. The thus constructed plasmid was introduced into *Pichia pastoris* GTS115 to obtain a transformant UHG42-3 (JP-A-4-299984 or EP-A-506040).

(2) Medium composition

YPD medium (2% Bacto-peptone, 1% yeast extract and 2% glucose) was used for the seed culture. A MeOH-ammonium acetate medium shown in Table 1 was used in the main culture.

Table 1

Composition of MeOH-ammonium acetate medium	
Component	Concentration (mg/l)
Methanol	40 ml
Glycerol	1,000
CH_3COONH_4	5,000
KH_2PO_4	10,000
$CaCl_2$ $2H_2O$	100
KCl	2,000
NaCl	100
$MgSO_4$ $7H_2O$	2,000
$ZnSO_4$ $7H_2O$	100
$CuSO_4$ $5H_2O$	5
$FeCl_3$ $6H_2O$	100
Biotin	0.1
Vitamin B_1	10
Vitamin B_6	1
Sodium pantothenate	10
Inositol	50

(3) Culturing method

i) Seed culture

A 1 ml portion of the strain contained in a freeze-dried stock vial was inoculated into a 300-ml baffled Erlenmeyer flask containing 50 ml of YPD medium and cultured at 30 • C for 24 hours with shaking.

ii) Main culture

A 1 ml portion of the se d culture m dium was inoculat d into a 300-ml baffled Erlenmeyer flask containing 50 ml of the MeOH-ammonium acetate medium which had been supplemented with each amino acid to give a final concentration of 0.1% and adjusted to pH 6.0, and cultured at 30 • C for 89 hours with shaking.

REFERENCE EXAMPLE 1

Measurement of cell density

During the main culturing carried out in Example 1 (3) ii), the culture broth was sampled at optional intervals, each of the thus collected samples was diluted with distilled water to give the OD_{540} value of 0.3 or less at the time of measurement, and then absorbance of the diluted sample at 540 nm was measured using a spectrophotometer (UV 200, manufactured by Shimadzu Corp.).

REFERENCE EXAMPLE 2

Evaluation of rHSA productivity

The entire portion of the culture broth after completion of the culturing was recovered and subjected to 20 minutes of centrifugation at 3,000 rpm. The resulting supernatant was clarified by filtering it through MILLEX-HV (Millipore Corp.; 0.45 μm), and a 15 ml portion of the resulting filtrate was concentrated about 20-fold using Amicon CentriPrep 10 (molecular weight cutoff of 10,000, manufactured by Amicon Corp.) (4°C, 3,000 rpm, about 6 hours) and then subjected to HPLC gel filtration analysis under the following conditions to evaluate HSA productivity:

Column : TSK gel $G3000SW_{x1}$ (Tosoh Corp.)
Mobile phase: 0.3 M NaCl, 50 mM Na-Phosphate, 0.1% NaN_3, pH 6.5
Flow rate : 0.7 ml/min
Injection : 50 μl
Detection : A_{280}, A_{350} (dual wave length)

REFERENCE EXAMPLE 3

Evaluation of coloring degree of produced HSA

A_{350}/A_{280} values were calculated using the results of the HPLC gel filtration analysis carried out for the evaluation of HSA productivity, and these values were used for the evaluation of coloring degree of HSA produced by the process of the present invention.

TEST EXAMPLE 1

Effects of each amino acid contained in the medium were examined. Amino acid content of the medium was fixed to 0.1 w/v %. Other conditions including culture conditions were as described in Example 1. The results are shown in Tables 2 to 4. Productivity of HSA, amount of cells and degree of coloring are respectively shown by percentage based on the case of no amino acid addition as 100%.

Table 2

Amino acid and other components	Yield (%)	Cell Yield (%)	Coloring degree (%)
Control	100	100	100
Yeast extract	157	118	127
Peptone	122	120	122
Gly	127	123	145
Ala	220	99	112
Asp	258	111	135
Arg	132	98	125
Glu	173	109	145
His	410	76	96
Ile	144	105	118
Lys	42	83	104
Met	28	24	124

Table 3

Amino acid and other components	Yield (%)	Cell yield (%)	Coloring degree (%)
Control	100	100	100
His	439	65	72
Lys	21	44	97
Ala	146	103	109
Asp	221	107	131
Trp	238	91	215
Val	235	87	102
Leu	117	102	111
Ser	200	91	105
Thr	105	103	137
Asn	119	121	139
Gln	78	113	105
Pro	114	115	135
Ca pantothenate	72	106	120

Table 4

Amino acid	Yield (%)	Cell yield (%)	Coloring degree (%)
Control	100%	100%	100%
Phe	165	89	112
Cys	134	98	161

TEST EXAMPLE 2

Effects of amino acid concentrations (0.01 to 1 w/v %) in the medium were examined. His was used as the amino acid. Other conditions including culturing conditions were as described in Inventive Example 1. The results are shown in Table 5.

Table 5

Amino acid	Concentration % (w/v)	Yield (%)	Cell Yield (%)	Coloring degree (%)
Control		100	100	100
His	0.1	326	82	112
His	0.2	214	67	88
His	0.3	188	86	71
His	0.4	185	89	71
His	0.6	159	71	76
His	1.0	133	85	71

As is evident from the above test results, yield of HSA can be increased significantly when an HSA-producing host is cultured in a medium which contains an amino acid, especially Ala, Asp, Glu, His, Phe, Trp, Val or Ser. When compared with an amino acid-free culture system, it was found that the HSA yield-increasing effect of a medium which contains Ala, His, Phe, Trp, Val or Ser or a medium which contains Arg or Cys was not due to an increase in the cell yield.

According to the present invention, yield of HSA by an HSA-producing host prepared by gene manipulation techniques can be incr ased by mploying a process which can be carried out easily with a relatively low cost by simply changing culture conditions. In particular, the HSA production yield can be increased 1.5 to 5 times in comparison with the cas of culturing with no supplement of amino acids, when cultured using a medium which contains at least one amino acid selected from Ala, Asp, Glu, His, Phe, Trp,

Val and Ser. In addition, according to a process which uses a medium supplemented with at least one amino acid selected from Ala, His, Phe, Trp, Val, Ser, Arg and Cys, the HSA production yield can be increased 1.3 to 5 times independent of the cell growth. Such an effect is particularly significant in a His-containing medium. Also, the HSA productivity-increasing ffect can be obtained even with a relatively small amino acid content of 0.08 to 1 w/v %. In addition, some of the above-described amino acids can reduce coloring of the produced HSA.

On the basis of these effects, the HSA production process of the present invention can be regarded as practically useful.

While the instant invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

Claims

1. A process for producing recombinant human serum albumin which comprises culturing a human serum albumin-producing host, prepared by gene manipulation techniques, in an amino acid-containing medium.

2. The process for producing recombinant human serum albumin according to claim 1 wherein said medium contains at least one amino acid selected from the group consisting of alanine, aspartic acid, arginine, cysteine, glutamic acid, histidine, isoleucine, phenylalanine, serine, tryptophan and valine.

3. The process for producing recombinant human serum albumin according to claim 1 wherein said medium contains at least one amino acid selected from the group consisting of alanine, aspartic acid, glutamic acid, histidine, serine, phenylalanine, tryptophan and valine.

4. The process for producing recombinant human serum albumin according to claim 1 wherein said medium contains at least one amino acid selected from the group consisting of alanine, aspartic acid, histidine, serine, tryptophan and valine.

5. The process for producing recombinant human serum albumin according to claim 1 wherein said medium contains at least one amino acid selected from the group consisting of alanine, arginine, histidine, phenylalanine, serine, tryptophan and valine.

6. The process for producing recombinant human serum albumin according to claim 1 wherein said medium contains histidine.

7. The process for producing recombinant human serum albumin according to claims 1-6, wherein said medium contains said at least one amino group in an amount of 0.08 to 20 w/v%.

8. The process for producing recombinant human serum albumin according to claim 7, wherein said medium contains said at least one amino group in an amount of 0.1 to 1.0 w/v%.

9. The process for producing recombinant human serum albumin according to claims 1-8, wherein said human serum albumin-producing host is a microorganism or an animal cell.

10. The process of claim 9 wherein said microorganism is a yeast strain.

11. The process of claim 9 wherein said microorganism is selected from the group consisting of *Escherichia coli*, a yeast strain, and *Bacillus subtilis*.

12. The process of claim 10 wherein said yeast strain is a member of the genus *Saccharomyces*, *Pichia* or *Hansenula*.

13. The process of claim 12 wherein said member of said genus *Saccharomyces* is *Saccharomyces cerevisiae* AH22.

14. The process of claim 12 wherein said member of said genus *Pichia* is *Pichia pastoris* GTS115.